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Diffusion of Gases through Plant Tissues[1]

ENTRY OF ACETYLENE INTO LEGUME NODULES

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ABSTRACT

I have measured acetylene diffusion through plant tissues including nodules from several species of legume—vetch, peas, soybeans, and *Sesbania rostrata*. The observed half-time for reequilibration of internal and external concentration is less than 1 minute for typical nodules. Inward diffusion of acetylene in air is rapid relative to the use of acetylene by nitrogenase so that diffusion of acetylene would not be a significant limiting factor for nitrogenase activity in air. However, under an atmosphere of Ar:O_2 where there is no N_2 reduction, the inward diffusion rate of acetylene into larger nodules could produce a measurable limitation of observed nitrogenase activity at low acetylene concentrations.

The diffusion of gases through plant tissues has been of interest for decades in attempts to understand the functioning of those enzymes such as ribulose bisphosphate carboxylase and nitrogenase that use gaseous substrates (7). Sinclair and Goudriaan (9) examined the possibility of diffusion limitation in the oxygen requirement for nitrogen fixation in legume root nodules. They reasoned that because about six oxygens are required for each nitrogen fixed and they have identical diffusion coefficients, oxygen rather than nitrogen must be limiting for nodule function. More recently, Denison *et al.* (4) have suggested that during acetylene reduction assays in the intact plant under air there may be a diffusion limitation of observed acetylene reduction. Similar studies were performed by Winship and Tjepkema (12) using an actinomycete symbiosis under Ar:O_2, who found that to fit their data it was necessary to assume a diffusion limitation term. Therefore, I have made direct measurements of the rate of acetylene diffusion through plant tissues, especially legume nodules. The inhibition that Denison *et al.* (4) observed at low levels of acetylene was primarily due to competitive inhibition by nitrogen and not to limiting acetylene diffusion. However, under some conditions (*e.g.* large nodules, low acetylene, argon atmosphere combined), diffusion limitation may be significant.

MATERIALS AND METHODS

Hairy winter vetch was grown outside in sandy soil inoculated with a commercial inoculum (Nitragin Co.). Plants were dug March 10th when air temperature was 3°C. Soybeans (Williams, inoculated with *R. japonicum* USDA strain 110) were grown in a growth chamber for 4 weeks under conditions of 28°C days and 22°C nights with a daylength of 16 h. Peas (Alaska, inoculated with *R. leguminosarum* 92A83) were grown under similar conditions for 23 d. *Sesbania rostrata* were grown from cuttings and inoculated with strain ORS 571. They were maintained under continuous light at approximately 30°C for 5 months. Apples (Golden Delicious and Jonathan) were obtained at a local market after several months of controlled atmosphere storage. *Bryophyllum tubiflorum* was grown in a south-facing laboratory window and kept well watered.

Acetylene was freshly generated from CaC_2. Argon was from Matheson Gas Products, while a mixture of 80% Ar:20% O_2 was from Air Products. Assays were done at 18 to 20°C. GC was carried out as previously described (2) with N_2 carrier gas using a column of Porapak R for acetylene and ethylene, by flame ionization, and Porapak N for measurement of Ar by thermal conductivity.

Several different techniques were used for measuring the rates of acetylene diffusion through plant tissues. The basic principle in each of these was to measure a relaxation process, influx or efflux of gas, in which the plant tissue constituted a significant fraction of the total volume of the test container. The most convenient method is as follows. A disposable plastic syringe was fitted at the inside bottom with a porous polyethylene disc held in place by an O ring. A small serum stopper was applied on the luer tip of the syringe. Plant tissue was added to fill the syringe approximately two-thirds full. The upper end of the syringe was then stoppered tight with a vaccine bottle stopper. Following release of excess pressure by a syringe needle, acetylene was added. The syringe was shaken and 50-μl samples were taken at intervals. The acetylene and ethylene were measured by GC until equilibrium was attained. Then the syringe was opened at both ends and a vacuum was applied to the luer end. After 15 s, the vacuum was removed, the syringe was quickly restoppered, and samples were again taken at intervals.

I estimated the interstitial volume of *S. rostrata* nodules in the following way. Fresh nodules, after use for acetylene reduction assays or ethylene diffusion, were placed in unstoppered syringes and purified argon was passed over them slowly from below for 0.5 h. Then they were quickly flushed with pure N_2 from below for 15 s; then, after both ends were stoppered, the rate and extent of Ar efflux was measured. Apple sections were treated in a similar way. As a further check of the relaxation technique and diffusion calculations, I measured the diffusion of acetylene into and out of thin layers of 2% agar poured on both flat sides of plastic tissue culture flasks (25 cm^2 surface area of one side). The flasks were modified by making a hole plugged with a serum stopper opposite the screw cap top so that direct flushing was possible.

RESULTS AND DISCUSSION

Acetylene and Ethylene Flux Rates. Some typical results for efflux of acetylene and ethylene are shown in Figure 1. Apparent

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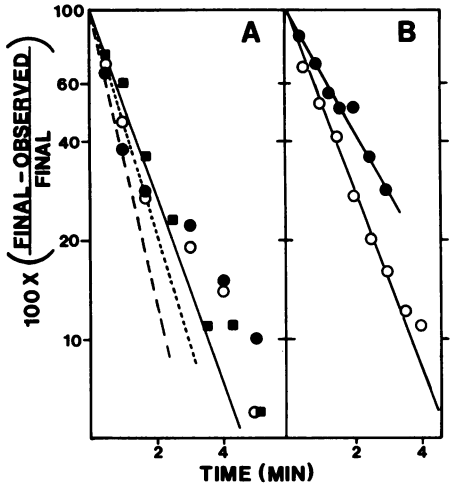

FIG. 1. The $t_{½}$ values for acetylene and ethylene diffusion out of nodules. For this experiment, I used nodules that had grown during 6 weeks on 6-month-old *S. rostrata* plants. For part A, stem nodules were used (2.8 mm average diameter), placing 0.76 g nodules in a 3-ml syringe (actual final gas voume, 3.5 ml). Acetylene (0.3 ml) was added and sampling was done as described in "Materials and Methods". Results of three flushings are shown as ●, O, and ■, respectively, plotting 100% of final acetylene level over final level on a log scale. Part B shows ethylene efflux from *S. rostrata* root nodules after the first flushing. Nodules were sorted into two size classes of less than and greater than 2.4 mm minimum dimension (mean number equivalent sphere diameter, 2.4 and 3.0 mm), shown by O and ●, respectively. Nodules were equilibrated with ethylene at ⅕ atm for 30 min prior to a 15-s flush with air. Five-ml syringes (6.5 ml gas volume) were used with 2.3 and 2.1 g of large and small nodules, respectively.

half-times for equilibration derived by the method shown in Figure 1 are presented in Table I. When a winter vetch was used for acetylene reduction assays, there was a decrease in flux rates after 2 d storage of the nodules at 0°C in the presence of acetylene. This increased flow resistance may be the same type as reported by Witty *et al.* (14) for pea, alfalfa, and clover, and which they attributed to nitrogen starvation of the nodule.

An alternative approach to estimating the impact of diffusion on acetylene reduction is to measure the activity at short intervals and to determine the extent of the lag phase before the rate of measured enzyme activity becomes linear. Using high concentrations of enzyme (that is, a large volume fraction of nodules in the assay vial), one can measure activity over very short time intervals. Some typical results for the increasing rate observed on first addition of acetylene are shown in Figure 2. In similar fashion one can determine the $t_{½}$ to a decreased rate following flushing of the reaction vessel with air. The $t_{½}$ values obtained in this way are maximal because they depend on the flux rates of both substrate and product. Depending on the substrate level used, the rate of enzyme action may be nearly proportional to available substrate (at low levels) or almost independent of it (at high concentrations).

To test whether gas diffusion in legume nodules is typical of plant tissues in general, I measured the rate of equilibration for some other tissues by the same method described above. Apple sections behaved in a manner very similar to nodules ($t_{½}$, ~50 s) while *Bryophyllum* leaves behaved as highly impermeable ($t_{½}$, ~2 min before and 3 min after water stress). Half-times for acetylene diffusion out of agar layers were 2 to 3 min for 1 mm thick and 4 to 8 min for 2 mm thick layers compared to calculated times of 1.9 and 7.6 min (see below).

Argon Flux Rates and Interstitial Gas Volume. The calculated interstitial gas volume of *S. rostrata* nodules on the basis of argon efflux to equilibrium was 3.5% for the stem nodules and 5.0 and 4.8% for large and small root nodules, respectively. For apple, the estimated value was 20% of the total volume, and for water-stressed *Bryophyllum* leaves it was 8%. The $t_{½}$ for argon efflux from stem nodules (50 s) is about that expected for free diffusion of gas through spheres of that size while that for root nodules (90 and 125 s for small and large nodules) is much slower, slightly slower than acetylene efflux from the same nodules (Table I). The difference in efflux rates between stem and root nodules is explicable if the interstitial gas of stem nodules is located in the outer layer of photosynthetic tissues as well as in the bacteroid-containing tissue and if there is no significant barrier to gas flow. The longer $t_{½}$ for root nodules suggests that there is a barrier (11) to gas flow in root nodules external to the interstitial gas. The estimated fraction of interstitial gas may be a slight underestimate due to the time (15 s, or less than one-third of a $t_{½}$) used for flushing the sample chamber.

Argon should provide a good surrogate for oxygen because their relative solubilities in aqueous and lipidic medium and diffusion coefficients are quite similar (10). Unfortunately, these two gases are not well resolved on my GC system so I cannot very easily study the diffusion of argon in the presence of atmospheric levels of oxygen. Nor can one study oxygen diffusion in nodules directly because it is rapidly consumed, once it enters. Because the nodules are necessarily anaerobic when under argon,

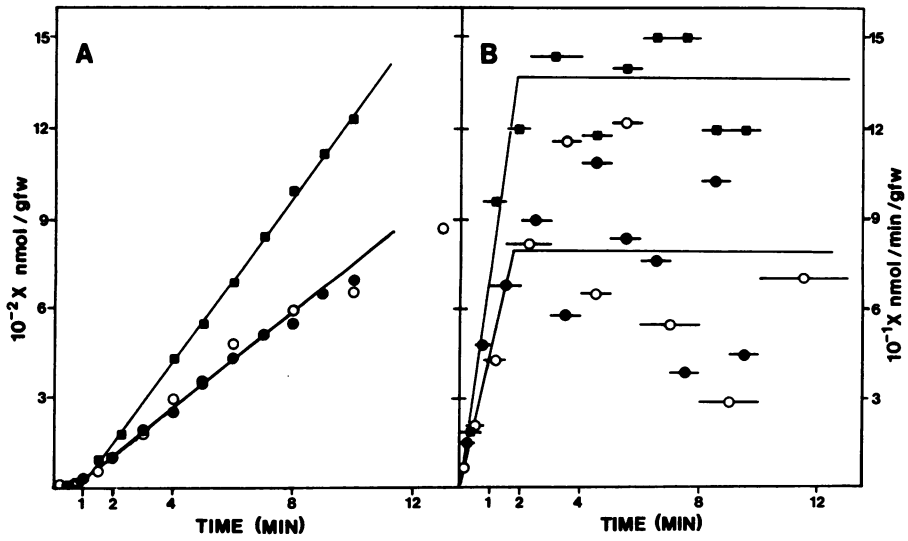

FIG. 2. Lag times during acetylene reduction by legume nodules. Part A shows the ethylene accumulation rate and part B the differential rate of ethylene production by stem nodules of *S. rostrata* (the same as shown in Fig. 1) and by large (3 mm diameter) or small (2 mm diameter) root nodules. Symbols are: (●) large root nodules; (O) small root nodules; (■) stem nodules. In part B, the horizontal lines show the best straight line rates obtained in part A. Bars on data points indicate the time intervals over which the observed rates were determined. The $t_{½}$ for either part lies between 45 s and 1 min.

Table I. *The $t_{½}$ Values for Equilibration of Acetylene with Legume Nodules*

Nodule Source	Influx	Efflux	Dilution by Flushing	Lag Time to[a] Steady State	Expected[b] Flux Rate
			s		
Vetch (freshly picked, average 2 mm diameter)	15 ± 1 (3)[c]	35 ± 10 (5)	13 ± 2 (5)	35 ± 5 (3)	17
Vetch (2 d cold with C_2H_2)	90	70, 55	15, 25	45, 60	17
Peas		60 ± 15 (3)			
Soybean (average 2.3 mm diameter)	35, 45	40, 50	43 ± 6 (3)	35 ± 5 (3)	23
S. rostrata, stem and root (average ~3 mm diameter)	80	65 ± 10 (3)	50 ± 10 (3)	45	39
S. rostrata, stem and root (average ~2 mm diameter)	70	40, 60	35, 35	50	17
S. rostrata, stem (average 2.8 mm diameter)	95	50 ± 10 (3)	40, 30	60	34
S. rostrata, root[d] (>2.4, mean 3.0 mm diameter)		98			39
S. rostrata, root[d] (<2.4, mean 2.4 mm diameter)		70			24

[a] Derived from plot of relative rate *versus* time.

[b] Calculated for diffusion into a sphere of indicated radius corresponding to the number mean diameter of the nodule population. For nodules of 0.5, 1.0, 1.5, 2.0, and 5.0 mm radius, the $t_{½}$ values for the nodule as a whole are 4.3, 17, 39, 68, and 432 s while the $t_{½}$ values for the center are 19.7, 78, 179, 313, and 1968 s.

[c] Mean ± SD for *n* in parentheses.

[d] Measured with ethylene rather than acetylene.

the rate of argon efflux under nitrogen may not fairly represent the rate of gas exchange that occurs in an aerobic system. However, the measured interstitial volume ought not to be dependent on the efflux rate. Dixon *et al.* (5) estimated the intercellular space at about 1% for pea and lupine nodules less than 2 mm in diameter. The estimate of 3 to 5% interstitial space in *S. rostrata* nodules is close to the 2.5 to 5% found by Bergersen and Goodchild (1) for soybean nodules.

Nodule Number *versus* Nodule Mass. Because the characteristic half-time of equilibration depends on the square of the nodule radius, population size distribution is important in determining $t_{½}$ values. In addition, volume varies with the cube of the radius so that for an equal number of nodules of two different sizes, the mass amount is strongly dominated by the larger nodules. In an experiment using preserved soybean nodules from a field study, I found that 58% of the total nodule mass was in nodules between 2.4 and 3.0 mm diameter, while only 35% of the total number (750 from nine plants) were in this size range. The calculated (see below) $t_{½}$ for equilibration using the mean mass size of 2.7 mm diameter is 30 s which is 1.5 times longer than that calculated from the mean number size of this population. Thus, a relatively small number of large nodules will dominate diffusion effects measured in a population of nodules. For a normal distribution of nodule sizes, the extent of skewing due to volume effects can be precisely predicted. However, nodule sizes are not normally distributed, but reach a limiting size before senescence. This may be because diffusion rates set a definite upper bound on functional nodule size. With white clover, we noted a considerable range of typical maximum nodule sizes, varying among seedlings of a single cultivar (3), suggesting some genetic control of size.

Models and Calculations. Sinclair and Goudriaan (9) proposed that oxygen diffusion into nodules must be limited by a thin layer of high resistance surrounding a region of higher permeability. They calculated that without the assumption of relatively high internal permeability, the center of nodules would become anaerobic because of the high rate of oxygen consumption. For acetylene, the diffusion limitation is obviously less important because about two O_2 are used for each acetylene reduced (9). The solubility coefficient of acetylene in water is about 1 at the range of temperatures of interest (10), so that the equilibrium concentration of acetylene is approximately 200-fold higher than that of O_2, giving a significantly greater driving force for diffusion through the presumably aqueous barrier layer to the internal parts of the nodule.

The rate of diffusion of acetylene through an aqueous medium may be estimated from the diffusion coefficient in water, 1.76×10^{-5} cm^2/s, cited by Jost (6). The value given is very close to that assumed by Sinclair and Goudriaan (9) for O_2. A recent chapter by Rose (7) provides formulae and graphs for calculating diffusion equilibration $t_{½}$ values for spheres and cylinders as well as slabs. This work assumes that external levels of the diffusing substance are constant, while the work of Jost (6) deals with a relaxation process across a boundary. For our experiments the external concentration does not remain constant, but for most typical experimental set-ups this would be a valid assumption. Diffusion into a sphere is 6.5 times faster than into one side of a slab and twice as fast as into a cylinder of the same radius (roughly in proportion to the number of directions from which a diffusing substance can enter). Solving the equations gives expected half-times shown in a footnote to Table I for spheres. It takes 4.6 times longer for the center of the sphere to reach half the external concentration than for the sphere as a whole, and 6 times longer for the whole sphere to reach 90% of the external concentration than to reach 50% of external. However, it takes only 1.5 times as long for the center to reach 90% of external as for the sphere as a whole because of nonlinearity in the concentration gradients.

The Role of Diffusion Limitation. It is possible to make some estimates of the extent to which diffusion of acetylene might limit observed activity. All of the experiments here described were done under an atmosphere of air with >75% N_2, a partial competitive inhibitor of acetylene reduction (2). Comparing the activity of nitrogenase under N_2:O_2 with that under Ar:O_2 at low

to moderate levels of acetylene allows one to estimate the extent to which N_2 inhibits nitrogenase at very low acetylene levels because of the approximately competitive nature of inhibition. From the work of Davis and Wang (2) and other (unpublished) studies on intact cow pea plants, this inhibition can be reliably estimated to be about 50% at 1/500 atm of acetylene. Thus, the activities observed in the present study underestimate the acetylene reducing potential by about 2-fold. From the observed rates of acetylene consumption, I calculate that in the absence of N_2 the most active fractions tested would consume <30% of the available internal acetylene per minute. With an assumed equilibration $t_{½}$ of 0.5 min, the steady-state decline in acetylene level would be just one-fourth the fractional rate of consumption, or <7.5%. At substrate levels significantly below the K_m, enzyme activity is directly proportional to substrate concentration, so there would be less than a 7.5% underestimate of enzyme activity.

Estimates of the possible diffusion limitation were made for soybean, vetch, and *S. rostrata* nodules using observed rates under air at acetylene levels from 0.18 to 12×10^{-3} atm. From the observed $t_{½}$ values for reequilibration and the fraction of acetylene consumed per minute under air, the calculated rate perturbation would be between 6% and 13% in the absence of N_2. This is small but measurable. Estimates are very sensitive to the assumed size distribution of the nodules being tested, with large nodules more substrate limited than small ones. The diffusion limitation effect for aqueous systems is 10-fold greater for 5-mm radius nodules than for 1.5-mm radius nodules. With large nodules such as those of Alnus (11), the diffusion limitation could be significant.

Nodules show a higher diffusion resistance than apple tissue since they give essentially the same $t_{½}$ and have significantly smaller dimensions than cylinders of apples 3.8 mm in diameter. The apple is somewhat faster to equilibrate than would be expected for an equivalent pure aqueous system (50 s *versus* 126 s calculated $t½$). Presumably this is because of the 20% air spaces that are less diffusion resistant than the fluid contained within the cells. Kalanchoë leaves, which are well known to be highly resistant to water flow, show a similarly high resistance to flow of acetylene, with a half-time ~2 times longer than calculated for a liquid cylinder of the appropriate dimensions, even though having 8% air spaces inside.

I have not yet applied this direct measurement technique to other tissues and other gases, but it would not be difficult to do so. Efflux of gases is more readily and reliably measured than influx, and since the diffusion rate on opposite sides of the boundary is independent of direction for a nonreacting substance it may reasonably be treated as a simple relaxation phenomenon. With gas *versus* aqueous phases on opposite sides of a boundary, the gas phase is always at equilibrium relative to the aqueous phase because the gas phase diffusion coefficient is ~10,000 times greater.

All of my analyses of penetration depth *versus* time have been based on an assumption of homogeneity and nonreactivity throughout the nodule which is not strictly valid. Nodules contain a cortical layer without nitrogenase and calculations of lag times in acetylene reduction are dependent on the thickness of this nonreacting layer. In stem nodules, the outer layer is photosynthetic tissue and may have a different structure and permeability from root nodules. Scott *et al.* (8) showed that if a boundary has greater resistance than the regions on either side of it, both sides tend to remain at equilibrium throughout their thickness and do not generate the gradients predicted from the equations given by Rose (7). Tjepkema and Yocum observed a high resistance boundary for soybean nodules (11). A more subtle complication is introduced when the diffusing substance reacts chemically on one side of the barrier. The net mass transfer can be greatly enhanced by such reaction, as for instance by binding of O_2 to leghemoglobin (13). Such binding leads to facilitated diffusion of the O_2, making invalid the use of any simple model for O_2 diffusion into root nodules. Consumption of O_2 by oxidases also steepens the effective diffusion gradient and enhances mass transfer through the nodule. The $t_{½}$ values that I have measured for equilibration are not model dependent, but mechanistic descriptions of the respiration and nitrogenase concentration dependence functions are. For nitrogenase, the fraction of acetylene used per minute is small enough that there ought never to be a zone of nodule depleted by more than 30 to 50% if the resistance inside the nodule is comparable to that of an aqueous system. Thus, the enzyme action will not be much decreased, nor will mass transfer be increased by perturbation of the diffusion gradient.

When using acetylene on tissues that respond to ethylene, we have to be concerned that significant ethylene-stimulated effects may be elicited even in the absence of nitrogenase. We have not yet studied acetylene diffusion rates under atmospheres other than air. There are clearly a number of interesting physiological questions yet to be explored.

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